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Principal Investigator: Laura Bowers

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Austin, TX 78712

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Laura Bowers

email: lwbowers@mail.utexas.edu

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14. ABSTRACT

In postmenopausal women, obesity is associated with a worse breast cancer prognosis, and this may be related to these patients' reduced response rate to aromatase inhibitor treatment. Obesity is accompanied by elevated levels of growth factors and inflammatory cytokines that can promote tumorigenesis and regulate the expression and activity of aromatase, the key enzyme in the synthesis of estrogen from androgens. The purpose of this project is to elucidate the role of local aromatase expression and activity in the promotion of breast cancer progression in obese postmenopausal women. Results have demonstrated that serum from obese postmenopausal women stimulates greater aromatase expression in adipose stromal cells (ASC) and MCF-7 breast cancer cells via direct and indirect mechanisms in comparison to serum from normal weight subjects. The obesity-induced aromatase expression in ASC and MCF-7 cells is associated with elevated breast cancer cell estrogen receptor alpha activity in the presence of exogenous testosterone. Further data indicates that the indirect effects of the obese sera on ASC aromatase expression are primarily mediated by inflammatory cytokine-induced prostaglandin E2 production by the cancer cells and macrophages. Based on these findings, future animal and human studies investigating the efficacy of an aromatase inhibitor/COX-2 inhibitor combination treatment in the obese postmenopausal population are being developed.

15. SUBJECT TERMS

Obesity, Aromatase, Interleukin 6, Prostaglandin E2, COX-2

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Introduction:

In postmenopausal women, epidemiological studies have shown that obesity increases the risk of developing breast cancer by approximately 40% (1, 2). It has also been established that obesity is associated with a worse breast cancer prognosis for both pre- and postmenopausal women, with the most significant effects seen in postmenopausal, estrogen receptor alpha (ERα) positive breast cancer (3-6). Following menopause, adipose tissue becomes the primary site of estrogen synthesis, which is catalyzed by the enzyme aromatase (7). Consequently, obese postmenopausal women have, on average, higher levels of circulating estrogens, leading to the speculation that elevated estrogen synthesis by the adipose tissue may be the primary mediator of breast tumorigenesis in this population (8). However, aromatization of androgens into estrogen also occurs in both the epithelial and adipose components of breast tissue, and examination of breast carcinoma tissue samples has revealed estrogen concentrations ten-fold greater than circulating plasma levels and two times the concentration found in normal breast tissue. This is correlated with a four to five-fold increase in aromatase expression within the breast tumor and tumor-bearing adipose quadrants (9). Thus, if increased estrogen levels are indeed driving greater breast cancer progression in obese postmenopausal women, it seems more likely that the key source of estrogen is local production within the mammary tissue, which obesity may be promoting. A recent study from Morris et al (10) supports this theory by showing that aromatase levels in the mammary tissue of breast cancer patients correlate with body mass index (BMI).

In addition to an increase in circulating estrogens, obesity is also associated with elevated levels of inflammatory cytokines and growth factors, and both are known to play a role in breast tumorigenesis (11-20). Specifically, the serum concentration of interleukin 6 (IL-6) is increased with obesity, and this inflammatory cytokine can promote aromatase expression both directly and indirectly in breast cancer epithelial cells and adipose stromal cells (ASC), the primary site of aromatase expression within the adipose tissue (21). Indirectly, Il-6 can enhance aromatase expression by promoting COX-2 expression in cancer epithelial cells and thereby inducing local production of prostaglandin E2 (PGE2), a potent stimulator of aromatase expression (21-22). Free insulin-like growth factor 1 (IGF-1), which is increased with obesity, may also be playing a role. This growth factor can regulate the expression and activity of aromatase, particularly in breast cancer epithelial cells (23-25). Taken together, this data led us to hypothesize that obesity-associated circulating factors may be driving local aromatase expression and activity even higher, thereby promoting greater local estrogen production and activity. The purpose of this project is to elucidate the role of local aromatase expression and activity in the promotion of breast cancer progression in obese postmenopausal women.

Body:

Task 1: Complete planned coursework that provides background and specific knowledge necessary for the PI's development as a breast cancer researcher with an emphasis on the role of nutrition (months 1-8).
All courses have been completed by the PI.

Task 2: Attend and present data at national conferences to enhance the PI's presentation skills, publicize her work, and provide opportunities for networking and discussion with other researchers regarding their work (months 1-36).
This task has been completed. See Appendix B for poster abstracts presented during the award funding period.

Task 3: Participate in the weekly seminar series hosted by the Department of Nutritional Sciences at UT Austin and the University of Texas MD Anderson Cancer Center-Science Park, which includes presentations by researchers from other institutions, departmental faculty, and departmental graduate students (months 1-36).
The weekly department seminar series was attended (Task 3a), and the PI presented her cumulative research findings at seminars in April 2012, January 2013, and October 2013 (Task 3b).

Task 4: Complete animal study for Specific Aim 1A (months 1-36).

Tasks 4a-d and 4f-j have been completed. All mice have completed study, and tissues and sera were collected as prescribed in the SOW. The last mice just completed study, so the DEXA and tissue/sera analyses for this task have not been done yet (Tasks 4e and 4k-n). However, some preliminary tissue analysis related to Task 6a has been completed (see below). Key findings from Task 4 include (See Appendix A for figures):

- Figure 1a: Mice receiving the high-fat diet-induced obesity (DIO) diet weighed significantly ($p<0.05$) more than those placed on the low-fat control diet at the end of each of the study's time points (the final average weight for each group excludes mice euthanized early due to tumor burden ≥1.5 cm).
- Figure 1b: Mice receiving the DIO diet mice had greater tumor incidence versus mice placed on the control diet in the 2-month and 4-month on diet groups, but not the 6-month group.
- Figure 1c: No difference in tumor latency between the DIO and control mice was seen in the 4-month and 6-month groups.
- Figure 1d: In the 4-month and 6-month groups, the DIO mice had a greater rate of tumor growth (mm/day) versus the control mice, though these differences did not reach statistical significance.

Task 5: Complete in vitro studies for Specific Aim 1B (months 13-36).

Tasks 5a-c and 5h have been completed. Key findings from the remaining sub-tasks under Task 5 include (see Appendix A for figures):

Task 5e:

- Figure 2: Ob serum stimulated greater Akt and ERK1/2 phosphorylation in MCF-7 and T47D cells after 15 minute and 1 hour exposures in comparison to Con serum ($p<0.05$).
- Figure 3: A 15 minute and 1 hour exposure to Ob serum stimulated greater ERα phosphorylation at ser167 (the Akt target site) ($p<0.05$), but not ser118 (the Erk1/2 target site), in MCF-7 cells vs Con sera.
- Figure 4: A 1 hour exposure to Ob serum did not promote more or less total ERβ expression in MCF-7 or ZR75 cells vs Con sera.

Tasks 5g and 5j:

- Figure 5: MCF-7 cells exposed to Ob serum for 24 hours had significantly greater aromatase expression, as measured by qPCR, in comparison to Con sera ($p<0.05$). The Ob serum-induced MCF-7 cell aromatase expression was significantly inhibited by the Akt II inhibitor and PD98059 (the MAPK inhibitor), but not LY294002 (the PI3K inhibitor). However, these same drug treatments all significantly increased Con serum-induced aromatase expression in the MCF-7 cells, possibly due to inhibition of negative feedback loops ($p<0.05$).
- Figure 6: Conditioned media (CM) from ASC exposed to Ob serum promoted significantly greater MCF-7 cell aromatase expression in comparison to CM from ASC exposed to Con serum ($p<0.05$). ASC CM was generated by exposing ASC to Ob or Con human serum for 3 hours, removing the serum and washing the cells with PBS, then incubating the ASC in serum-free media (SFM) for 24 hours. The SFM (now CM) was then collected and either used immediately to treat MCF-7 cells or frozen at -20°C for later use. The MCF-7 cells were exposed to the ASC CM for 24 hours. Neutralization of IL-6 in both the Ob and Con ASC CM (via the addition of an IL-6 antibody to the CM for 1 hour prior to its use as a treatment for the MCF-7 cells) significantly decreased the CM-induced MCF-7 cell aromatase expression, eliminating the difference between Ob and Con ($p<0.05$).
- Figure 7: There was no significant difference in the aromatase expression in ASC exposed to Ob serum vs Con for 24 hours.
- Figure 8A: CM from MCF-7 cells exposed to Ob serum induced significantly greater aromatase expression in ASC in comparison to CM from MCF-7 cells exposed to Con serum ($p<0.05$). MCF-7 cell CM was

generated by exposing MCF-7 cells to Ob or Con human serum for 1 hour, removing the serum and washing the cells with PBS, then incubating the MCF-7 cells in SFM for 24 hours. The SFM (now CM) was then collected and either used immediately to treat the ASC or frozen at -20°C for later use. The ASC were exposed to the MCF-7 CM for 24 hours. Inhibition of COX-2 activity in MCF-7 cells using celecoxib (30 uM) during the generation of CM with Ob and Con sera significantly reduced Ob and Con CM-induced ASC aromatase expression, eliminating the difference between Ob and Con ($p<0.05$). Neutralization of IL-6 in both the Ob and Con sera (via the addition of an IL-6 antibody to the sera for 1 hour prior to its use to generate CM) also significantly decreased the CM-induced ASC aromatase expression, again eliminating the difference between Ob and Con ($p<0.05$).

- Figure 8B: Ob MCF-7 CM that was generated as stated above contained approximately 13 times more PGE2 (as measured by ELISA) in comparison to Con MCF-7 CM ($p<0.05$), supporting our hypothesis that exposure to Ob sera enhances MCF-7 cell COX-2 activity in comparison to Con sera.
- Figure 9A: CM from U937 cells (matured from monocytes to macrophages with a 48 hour treatment of 10 ng/ml TPA) exposed to Ob serum induced significantly greater aromatase expression in ASC in comparison to CM from U937 cells exposed to Con serum ($p<0.05$). U937 cell CM was generated by exposing U937 cells to Ob or Con human serum for 1 hour, removing the serum and washing the cells with PBS, then incubating the U937 cells in SFM for 24 hours. The SFM (now CM) was then collected and either used immediately to treat the ASC or frozen at -20°C for later use. The ASC were exposed to the U937 CM for 24 hours. Inhibition of COX-2 activity in U937 cells with celecoxib (30 uM) during the generation of CM with Ob and Con sera significantly reduced Ob and Con CM-induced ASC aromatase expression, eliminating the difference between Ob and Con ($p<0.05$).
- Figure 9B: Ob U937 CM that was generated as stated above contained approximately 5 times more PGE2 (as measured by ELISA) in comparison to Con U937 CM ($p<0.05$), supporting our hypothesis that exposure to Ob sera enhances U937 cell COX-2 activity in comparison to Con sera.
- Figure 10: ASC exposed to Ob or Con CM from MCF-7 or U937 cells express at least 100-fold more aromatase in comparison to MCF-7 cells exposed to Ob or Con sera or CM from ASC, suggesting that local ASC in the breast may be the more biologically relevant source of aromatase versus the cancer epithelial cells.

<u>Task 6</u>: Complete tissue analysis and in vitro studies for Specific Aim 2, using the same panel of cell lines for the in vitro studies as listed for Aim 1B (months 9-36).

Key findings from the sub-tasks under Task 6 include (see Appendix A for figures):

Task 6a:

- Figure 11: The PI has not yet been able to complete any IHC analysis of the normal and tumor mammary tissue, but has measured the expression of 4 ERα target genes by qPCR in the tumor tissue. There was no significant difference between the DIO and control diet mice in tumor expression of pS2, PR, cyclin D1, or Bcl-2 (analysis of the data with the mice segregated according to diet time points did not change this result). Very low expression of pS2 in the tumors (indicated by high qPCR CT values for this gene, which is a very specific indicator of ERα activity), suggests that the tumors may not express ERα. Consequently, the PI plans to measure ERα expression in the normal and tumor mammary tissue next, but this data was not available in time for this report. If the tumors prove to be ERα negative, but the normal mammary tissue is ERα positive, then local aromatase expression could still be playing a role in early tumor promotion. However, the PI may also need to explore additional factors to explain the greater tumor incidence and growth rate seen with obesity in this study (see key findings from Task 4).

Tasks 6c and 6f:

- Figure 12A: MCF-7 and T47D cells exposed to Ob human sera for 24 hours did not demonstrate greater ERα activity in comparison to Con sera, as measured by qPCR analysis of pS2 expression. This indicates that any differences in circulating estrogen levels between obese and control subjects are not sufficient to induce a difference in breast cancer cell ERα activity.
- Figure 12B: MCF-7 and T47D cells exposed to Ob human sera for 24 hours did express 34% and 30% higher levels of cyclin D1, respectively, as measured by qPCR ($p<0.05$). This is likely due to estrogen-independent effects from other circulating factors that are increased in the Ob sera.
- Figure 13A and B: CM from a co-culture of MCF-7 cells and ASC exposed to Ob sera, then SFM with testosterone, promotes significantly greater ERα activity (measured by qPCR analysis of pS2 and cyclin D1 expression) in separate MCF-7 and T47D cells ($p<0.05$). MCF-7 cells were seeded on top of ASC, then the co-culture was exposed to Ob or Con sera for 1 hour. The sera was then removed and the cells washed with PBS, then incubated in SFM with testosterone (100 nM) +/- anastrozole (1 uM) for 24 hours. The media (now CM) was then collected and either used immediately to treat MCF-7 or T47D cells that had been seeded separately or frozen at -20°C for later use. The MCF-7 or T47D cells were exposed to the ASC/MCF-7 CM for 24 hours. This experiment tests whether the greater ASC aromatase expression induced by exposure to Ob MCF-7 CM versus Con (Figure 8A) then results in greater estradiol production that stimulates more breast cancer cell (MCF-7 or T47D) ERα activity. The difference seen between Ob and Con CM in MCF-7 cell pS2 expression is neutralized by the addition of anastrozole with the testosterone. However, for pS2 expression in T47D cells and cyclin D1 expression in both cell lines, aromatase inhibition significantly decreases Ob-induced expression, but not down to the level of Con with the aromatase inhibitor. This suggests that anastrozole may be less effective at inhibiting Ob versus Con sera-induced aromatase activity and estradiol production by the MCF-7 cells.
- Figure 14A and B: CM from a co-culture of U937 macrophages and ASC exposed to Ob sera, then SFM with testosterone, promotes significantly greater ERα activity (measured by qPCR analysis of pS2 and cyclin D1 expression) in MCF-7 and T47D cells ($p<0.05$). U937 cells were seeded on top of ASC and incubated in TPA (1 ng/ml) for 48 hours to differentiate the U937 monocytes to macrophages, then the co-culture was exposed to Ob or Con sera for 1 hour. The sera was then removed and the cells washed with PBS, then incubated in SFM with testosterone (100 nM) +/- anastrozole (1 uM) for 24 hours. The SFM (now CM) was then collected and either used immediately to treat MCF-7 or T47D cells or frozen at -20°C for later use. The MCF-7 and T47D cells were exposed to the ASC/U937 CM for 24 hours. This experiment tests whether the greater ASC aromatase expression induced by exposure to Ob macrophage CM versus Con (Figure 9) then results in greater estradiol production that stimulates more breast cancer cell (MCF-7 and T47D) ERα activity. The difference seen between Ob and Con here is neutralized by the addition of anastrozole with the testosterone ($p<0.05$).

Tasks 6e and 6f:

- Figure 15A: MCF-7 and T47D cells exposed to Ob human sera for 48 hours did not demonstrate greater ERα activity in comparison to Con sera, as measured by ERE luciferase assay. This indicates that any differences in circulating estrogen levels between obese and control subjects are not sufficient to induce a difference in breast cancer cell ERα activity.
- Figure 15B: There was still no difference in MCF-7 cell ERα activity, as measured by ERE luciferase, when subjects that were on aromatase inhibitor treatment at the time of sera collection were eliminated from the Ob and Con sera pools used to treat the cells for 48 hours. These results indicate that the lack of difference in breast cancer cell ERα activity following Ob versus Con sera exposure is not due to the use of aromatase inhibitor treatment by some subjects.
- Figure 16: When exogenous testosterone (100 nM) is added along with the sera to serve as the aromatase substrate, ERα activity is elevated in the MCF-7 cells exposed to Ob sera for 48 hours, as

measured by ERE luciferase. The simultaneous addition of the aromatase inhibitor anastrozole (1 uM) significantly decreased the ERα activity induced by Ob sera plus testosterone ($p<0.05$). These results indicate that the disparity in the aromatase expression (Figure 5) and subsequent estradiol production stimulated by Ob versus Con sera in these cells is large enough to promote a difference in ERα activity.

- Figure 17: CM from ASC exposed to Ob sera (versus Con), which was generated as described for Figure 6, induced greater MCF-7 cell ERα activity in the presence of exogenous testosterone (100 nM). ERα activity in the MCF-7 cells was measured by ERE luciferase following a 48 hour exposure to the ASC CM. The addition of anastrozole (1 uM) reduced ERα activity levels back to those seen with Ob CM alone ($p<0.05$). These results indicate that the disparity in the aromatase expression (Figure 6) and subsequent estradiol production stimulated by Ob versus Con ASC CM in these cells is large enough to promote a difference in ERα activity.
- Figure 18: CM from a co-culture of U937 macrophages and ASC exposed to Ob sera, then SFM with testosterone, promotes significantly greater ERα activity (measured by ERE luciferase assay) in MCF-7 and T47D cells ($p<0.05$). The CM was generated as described for Figure 14. The MCF-7 and T47D cells were exposed to the ASC-U937 CM for 48 hours. This experiment tests whether the greater ASC aromatase expression induced by exposure to Ob macrophage CM versus Con (Figure 9) then results in greater estradiol production that stimulates more breast cancer cell (MCF-7 and T47D) ERα activity. The difference seen between Ob and Con here was neutralized by the addition of anastrozole with the testosterone ($p<0.05$).

Key Research Accomplishments:

- Aromatase expression is elevated in MCF-7 cells exposed to Ob human sera and Ob CM from ASC in comparison to controls.
- This obesity-induced increase in MCF-7 cell aromatase expression results in greater MCF-7 cell ERα activity in the presence of exogenous testosterone, an effect that is decreased by the addition of an aromatase inhibitor.
- Aromatase expression is higher in ASC exposed to Ob CM from MCF-7 cells and U937 macrophages in comparison to controls; these effects are likely mediated by increased COX-2 activity and PGE2 production by both the MCF-7 cells and macrophages, as treatment of these cells with the COX-2 inhibitor celecoxib during the generation of CM neutralizes the difference in ASC aromatase expression between Ob and Con. Elevated IL-6 in the Ob sera is the likely mediator of the Ob sera-induced increase in MCF-7 cell PGE2 production and subsequent ASC aromatase expression, as IL-6 neutralization in the sera results in elimination of the difference between Ob and Con MCF-7 CM-induced ASC aromatase expression.
- This Ob CM-induced increase in ASC aromatase expression results in greater MCF-7 and T47D breast cancer cell ERα activity in the presence of exogenous testosterone, an effect that is neutralized by the addition of an aromatase inhibitor.

Reportable Outcomes:

Research manuscripts:

Bowers LW, Cavazos DA, Brenner AJ, Hursting SD, Maximo IXF, deGraffenried LA. Obesity enhances nongenomic estrogen receptor cross-talk with the PI3K/Akt and MAPK pathways to promote in vitro measures of breast cancer progression. Breast Cancer Research, 2013, 15:R59.

Bowers LW, Maximo IXF, Brenner AJ, Beeram M, Hursting SD, Price RS, Tekmal RR, deGraffenried LA. NSAID use attenuates breast cancer recurrence in obese women: Role of prostaglandin-aromatase interactions. Submitted to Cancer Research in December 2013.

Bowers LW, Brenner AJ, Hursting SD, Tekmal RR, deGraffenried LA. Obesity promotes elevated aromatase expression in the breast tumor microenvironment via upregulation of breast cancer cell prostaglandin E_2 production. In progress.

Conference posters:

Bowers LW, Maximo IXF, Brenner AJ, Beeram M, Hursting SD, Price RS, Tekmal RR, deGraffenried LA. NSAID use attenuates breast cancer recurrence in obese women: Role of prostaglandin-aromatase interactions. 36th Annual CTRC-AACR San Antonio Breast Cancer Symposium, 2013.

Bowers LW, Brenner AJ, Hursting SD, deGraffenried LA. Obesity promotes pre-adipocyte aromatase expression via breast cancer cell prostaglandin E2 production in an in vitro model of the breast tumor microenvironment. American Institute for Cancer Research Annual Research Conference, Bethesda, MD, 2013.

Bowers LW, Li R, Tekmal RR, Brenner AJ, Hursting SD, deGraffenried LA. Obesity promotes elevated aromatase expression in the breast tumor microenvironment via upregulation of prostaglandin E_2 production. Keystone Symposia: The Role of Inflammation during Carcinogenesis, Dublin, Ireland, 2012

Bowers LW, De Angel R, Brenner AJ, Tekmal RR, Hursting SD, deGraffenried LA. Obesity-associated growth factor signaling promotes aromatase expression and estrogen receptor activity in breast cancer cells. 102nd Annual Meeting of the American Association for Cancer Research, Chicago, IL, 2012.

Bowers LW, Brenner AJ, Li R, Tekmal RR, deGraffenried LA. Obesity-induced aromatase expression in the breast microenvironment promotes estrogen receptor activity independent of circulating estradiol levels. 34th Annual CTRC-AACR San Antonio Breast Cancer Symposium, 2011.

Bowers LW, De Angel R, Brenner AJ, Hursting SD, deGraffenried LA. Obesity-associated growth factor signaling upregulates aromatase expression in breast cancer cells. 101st Annual Meeting of the American Association for Cancer Research, Orlando, FL, 2011.

Funding/Scholarships:

- American Association for Cancer Research Scholar-in-Training Award, December 2013
- American Institute for Cancer Research Annual Research Conference Scholarship, November 2013
- Tyrrell E. Flawn Graduate Fellowship in Nutrition, University of Texas at Austin, May 2013

Employment/Research Opportunities:

The PI's defense is planned for May 2014, and she has accepted a postdoctoral position in the laboratory of Dr. Stephen Hursting, Department of Nutrition, University of North Carolina at Chapel Hill (Dr. Hursting will move to UNC from UT Austin in June 2014). The PI is applying for a Cancer Control Education Program Postdoctoral Fellowship through UNC's Lineberger Comprehensive Cancer Center and has been invited to interview for the fellowship.

Conclusion:

The results of this project indicate that circulating factors associated with obesity do promote greater aromatase expression in breast cancer cells and ASC via direct and indirect actions on the cells. The indirect mechanisms occur through paracrine interactions between these two cell types and between the ASC and macrophages. This obesity-induced elevation in aromatase expression is associated with an increase in ERα activity in the cancer cells. The data indicates that the relative aromatase expression levels are substantially higher in the ASC versus the cancer cells. Consequently, the PI has focused on exploring the pathways responsible for obesity-induced ASC aromatase expression. COX-2 inhibition in macrophages and MCF-7 breast cancer cells was shown to suppress obesity-induced ASC aromatase expression, paving the way for future animal and human studies investigating the efficacy of an aromatase inhibitor/COX-2 inhibitor combination treatment in the obese postmenopausal population. The ultimate goal of this project, the identification of a potentially more effective chemotherapeutic regimen for this patient population, has thus been achieved.

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Appendix A: Figures

Figure 1. (A) Weekly average weight of MMTV Wnt-1 mice in each time point (2, 4, and 6 months) and diet treatment (high-fat diet-induced obesity (DIO) versus low-fat control) group. (B) Percent tumor incidence in MMTV Wnt-1 mice maintained on the DIO versus control diet for 2, 4 or 6 months. (C) Tumor latency in MMTV Wnt-1 mice maintained on the DIO versus control diet for 2, 4, or 6 months (no tumors formed in the 2-month control group). (D) Tumor growth rate in MMTV Wnt-1 mice maintained on the DIO versus control diet for 2, 4, or 6 months. Asterisks indicate significant differences.

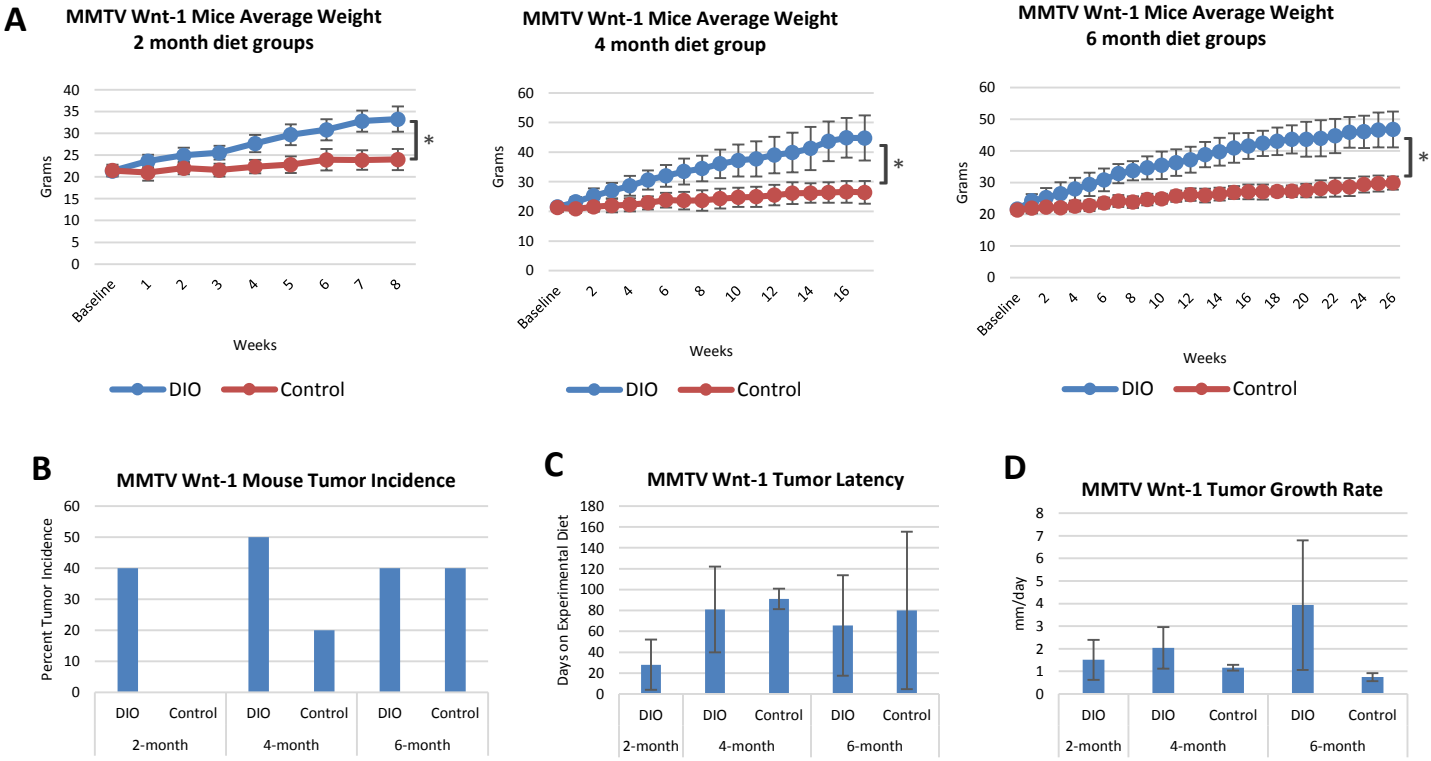


Figure 2. Akt (ser473) and ERK1/2 phosphorylation in MCF-7 (A) and T47D (B) cells after 15 minute and 1 hour of Ob or Con human sera exposure. Graphs represent pAkt (ser473) and pERK1/2 protein levels standardized to tAkt and tERK1/2 protein levels, respectively. Asterisks represent significant differences in comparison to Con ($p<0.05$).

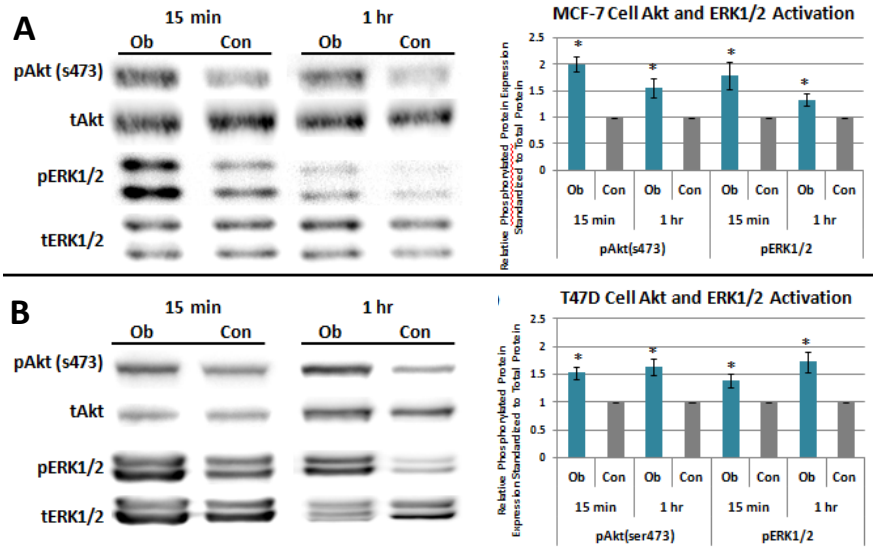


Figure 3. ERα phosphorylation in MCF-7 cells at ser167 (the Akt target site) and ser118 (the MAPK target site) after 15 minutes and 1 hour of Ob or Con human sera exposure. Graphs represent pERα protein levels standardized to tERα protein levels. Asterisks represent significant differences in comparison to Con ($p<0.05$).

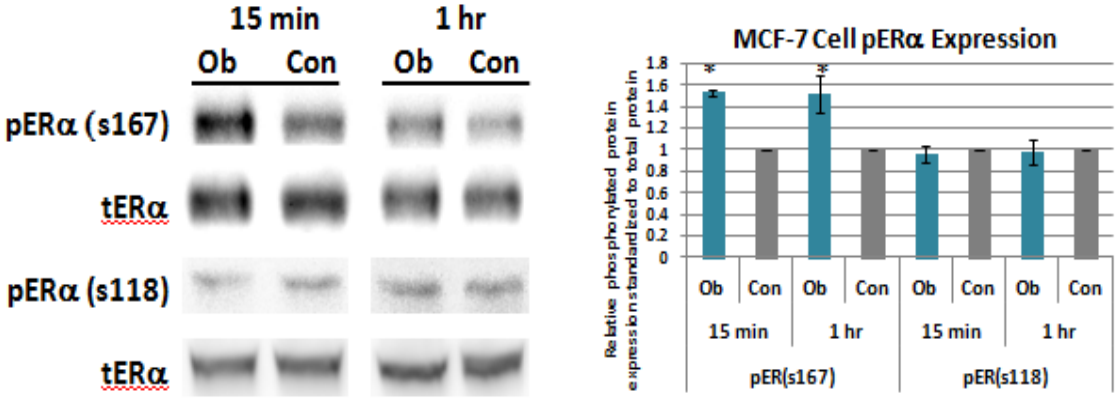


Figure 4. ERβ expression in MCF-7 and ZR75 cells was measured by qPCR following a 1 hour exposure to Ob or Con human sera. There were no significant differences between Ob and Con.

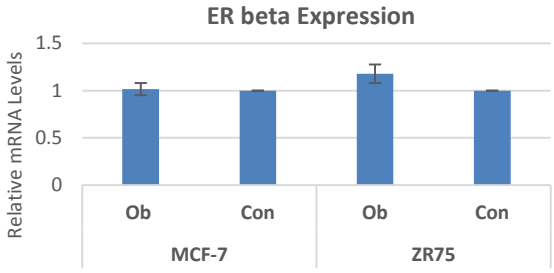


Figure 5. Aromatase expression was measured by qPCR in MCF-7 cells exposed to Ob or Con human serum for 24 hours, with or without the addition of the following inhibitors: LY294002 (LY, a PI3K inhibitor), Akt II inhibitor (AktII), and PD98059 (PD, a MEK inhibitor). Different letters indicate significant differences ($p<0.05$).

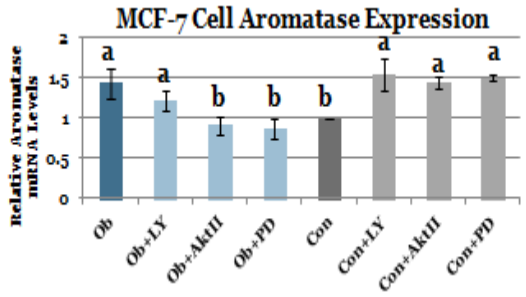


Figure 6. MCF-7 cell aromatase expression was measured by qPCR after a 24 hour exposure to ASC conditioned media (CM). IL-6 in the CM was neutralized by adding an IL-6 neutralizing antibody (Ab) to both the ObCM and ConCM. Different letters indicate significant differences ($p<0.05$).

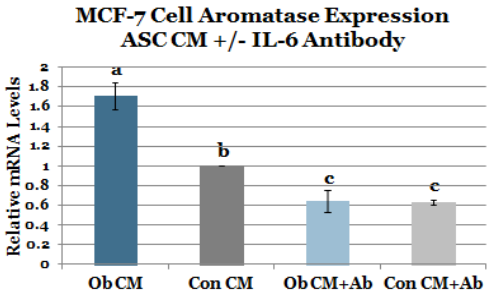


Figure 7. Aromatase expression in ASC exposed to Ob human serum versus Con for 24 hours was measured by qPCR. There was no significant difference between Ob and Con. Serum-free media (SF) and prostaglandin E2 (PGE2) were used as negative and positive controls, respectively.

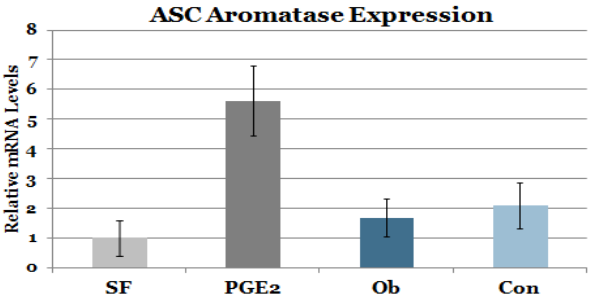


Figure 8. (A) Aromatase expression in ASC exposed to MCF-7 cell CM for 24 hours was measured by qPCR. Ob+C-CM and Con+C-CM indicate inhibition of COX-2 activity in the MCF-7 cells with celecoxib during the generation of CM with Ob and Con sera. Ob+AB-CM and Con+AB-CM indicate neutralization of IL-6 in both the Ob and Con sera (via the addition of an IL-6 antibody to the sera) prior to its use to generate MCF-7 cell CM. (B) MCF-7 cell CM was generated as stated above, and PGE2 levels in the CM were measured by ELISA. Different letters indicate significant differences and asterisks indicate a significant difference in comparison to control ($p<0.05$).

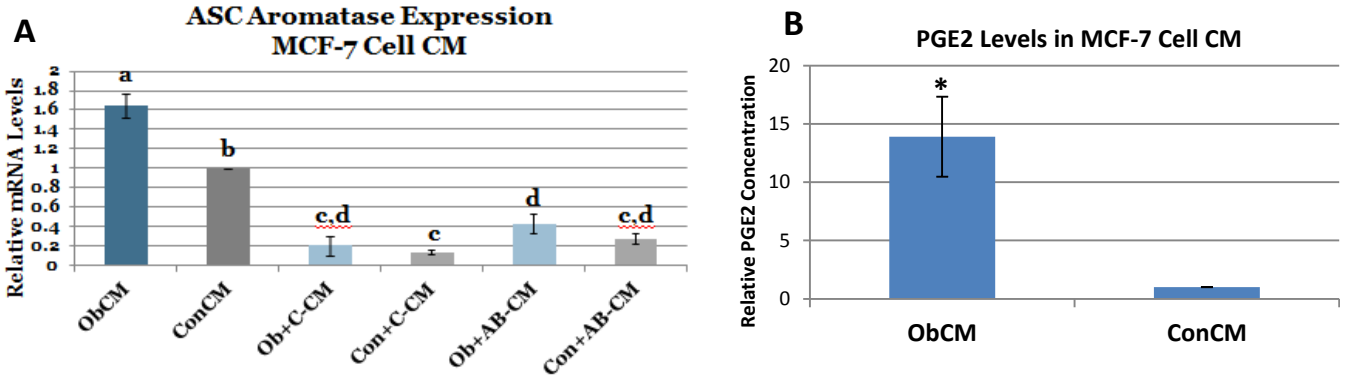


Figure 9. (A) Aromatase expression in ASC exposed to U937 cell (macrophage) CM for 24 hours was measured by qPCR. Ob+C-CM and Con+C-CM indicate inhibition of COX-2 activity in the U937 cells with celecoxib during the generation of CM with Ob and Con sera. (B) U937 cell CM was generated as stated above, and PGE2 levels in the CM were measured by ELISA. Different letters indicate significant differences and asterisks indicate a significant difference in comparison to control ($p<0.05$).

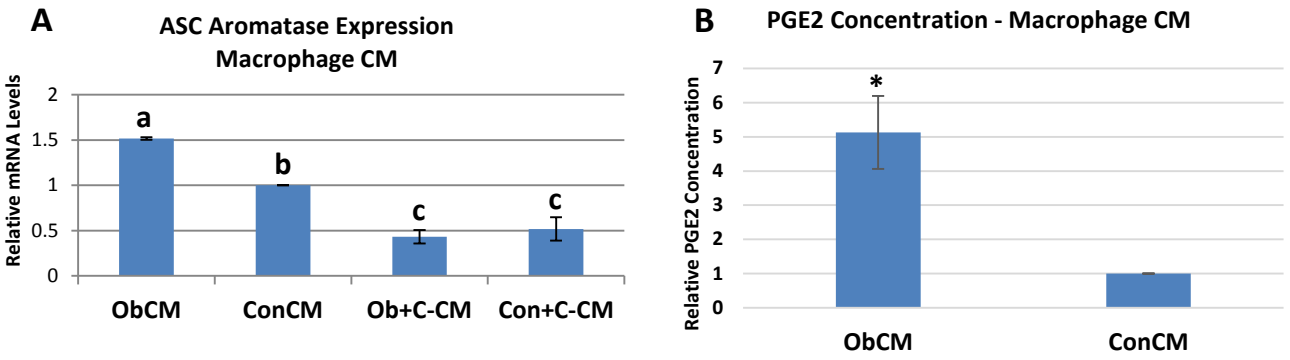


Figure 10. Aromatase expression in MCF-7 cells and ASC exposed to Ob or Con human sera or CM from various cell lines for 24 hours was measured by qPCR.

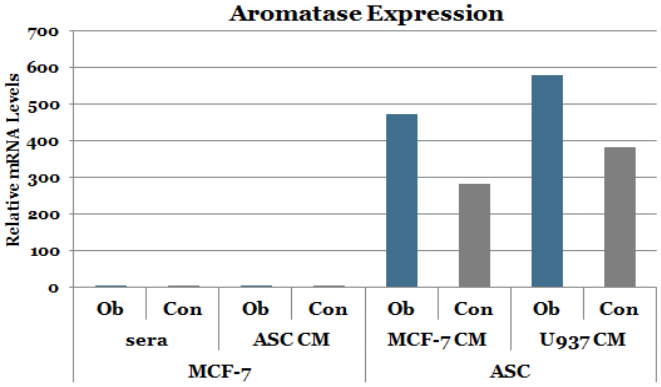


Figure 11. MMTV Wnt-1 mouse mammary tumor expression of ERα target genes, as measured by qPCR. Tumors from animals fed a high-fat diet-induced obesity (DIO) versus a low-fat control diet are compared. Genes measured include: (A) pS2, (B) progesterone receptor (PR), (C) cyclin D1, and (D) Bcl-2.

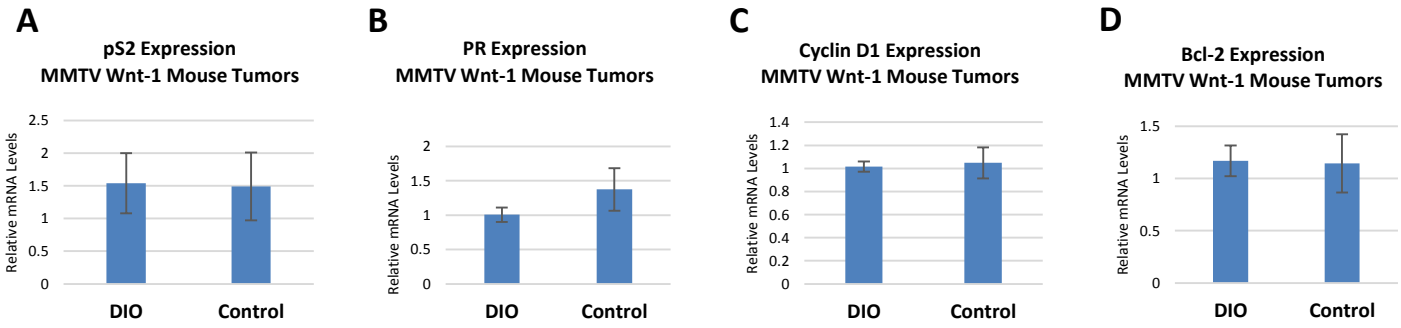


Figure 12. (A) ERα activity in MCF-7 and T47D cells exposed to Ob and Con human sera for 24 hours was measured by pS2 expression. (B) Cyclin D1 expression in MCF-7 and T47D cells exposed to Ob or Con human sera for 24 hours. Asterisks represent significant differences in comparison to Con ($p<0.05$).

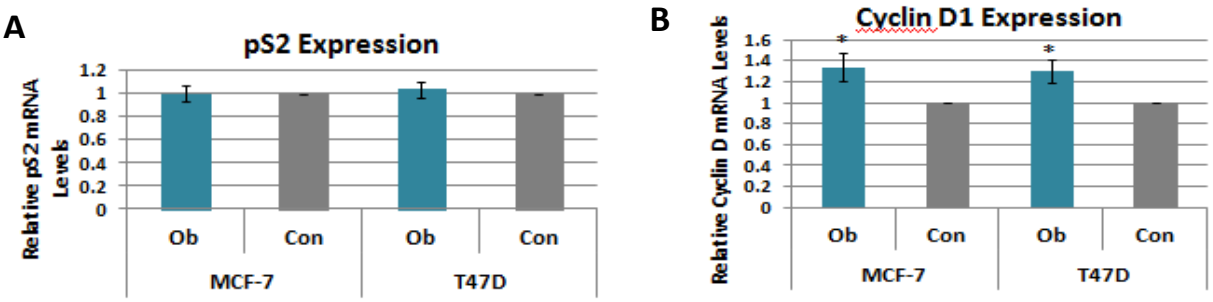


Figure 13. MCF-7 (A) and T47D (B) cell ERα activity, measured via qPCR analysis of pS2 and cyclin D1 expression, following a 24 hour exposure to CM from a co-culture of MCF-7 cells and ASC. This CM was generated by exposing the co-culture to Ob or Con human sera for 1 hour, removing the serum and washing the cells with PBS, then incubating the co-culture in SFM plus exogenous testosterone (ObCM and ConCM) or testosterone and the aromatase inhibitor anastrozole (Ob+AI-CM and Con+AI-CM) for 24 hours. The SFM (now CM) was then collected and used to treat separate MCF-7 or T47D cells. Different letters indicate significant differences ($p<0.05$).

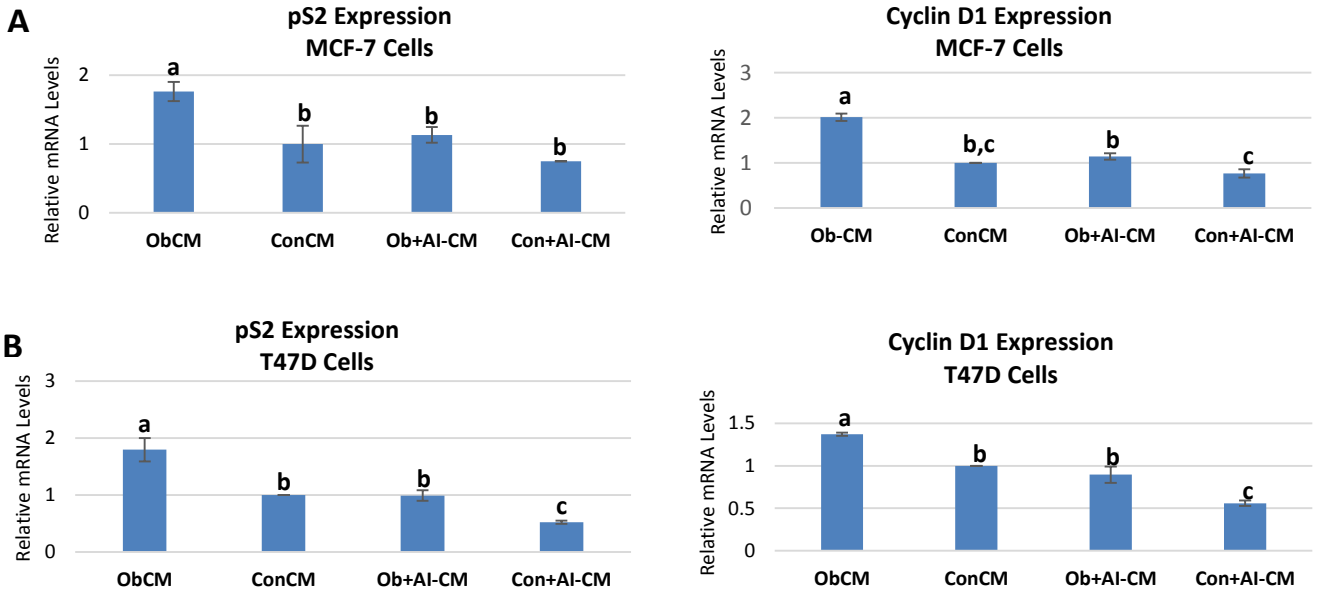


Figure 14. MCF-7 (A) and T47D (B) cell ERα activity, measured via qPCR analysis of pS2 and cyclin D1 expression, following a 24 hour exposure to CM from a co-culture of U937 cells (first differentiated to macrophages) and ASC. This CM was generated by exposing the co-culture to Ob or Con human sera for 1 hour, removing the serum and washing the cells with PBS, then incubating the co-culture in SFM plus exogenous testosterone (ObCM and ConCM) or testosterone and the aromatase inhibitor anastrozole (Ob+AI-CM and Con+AI-CM) for 24 hours. The SFM (now CM) was then collected and used to treat the MCF-7 or T47D cells. Different letters indicate significant differences ($p<0.05$).

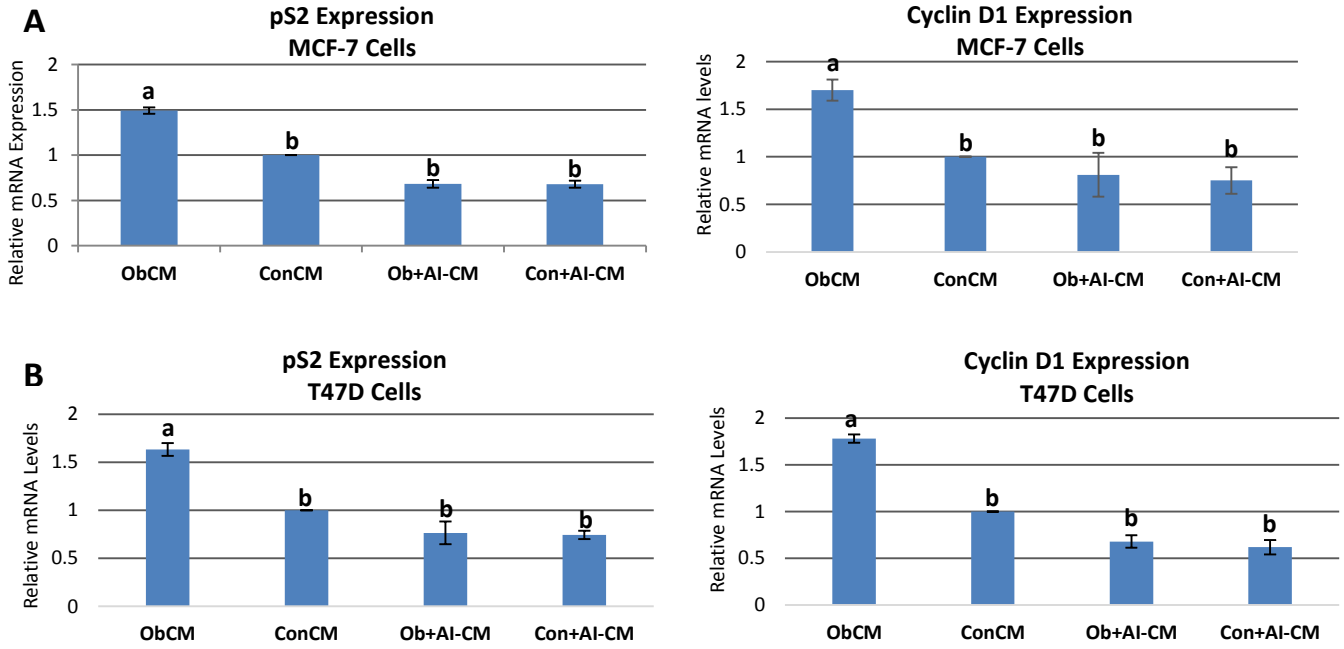


Figure 15. (A) ERα activity in MCF-7 and T47D cells exposed to Ob and Con human sera for 48 hours was measured by ERE luciferase assay. (B) ERα activity, as measured by ERE luciferase, in MCF-7 cells exposed to Ob or Con human sera. For this experiment, subjects that were on aromatase inhibitor treatment at the time of sera collection were eliminated from the Ob and Con sera pools (Ob(-AI) and Con(-AI)).

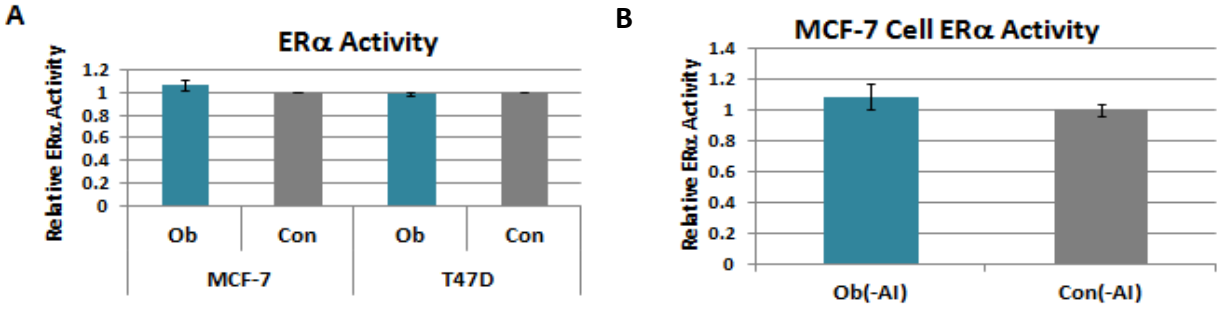


Figure 16. MCF-7 cell ERα activity, measured via ERE luciferase assay, following a 48 hour exposure to Ob or Con human sera alone, with exogenous testosterone added (Ob+T and Con+T), and with testosterone and the aromatase inhibitor anastrozole added (Ob+T+AI and Con+T+AI). Different letters indicate significant differences ($p<0.05$).

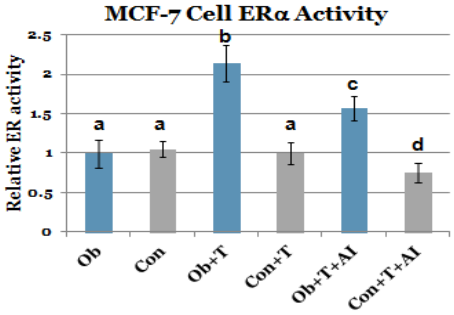


Figure 17. (A) MCF-7 cell ERα activity, measured via ERE luciferase assay, following a 48 hour exposure to ASC CM alone (generated with Ob or Con human sera as described previously), with exogenous testosterone added (ObCM+T and ConCM+T), and with testosterone and the aromatase inhibitor anastrozole added (ObCM+T+AI and ConCM+T+AI). Different letters indicate significant differences ($p<0.05$).

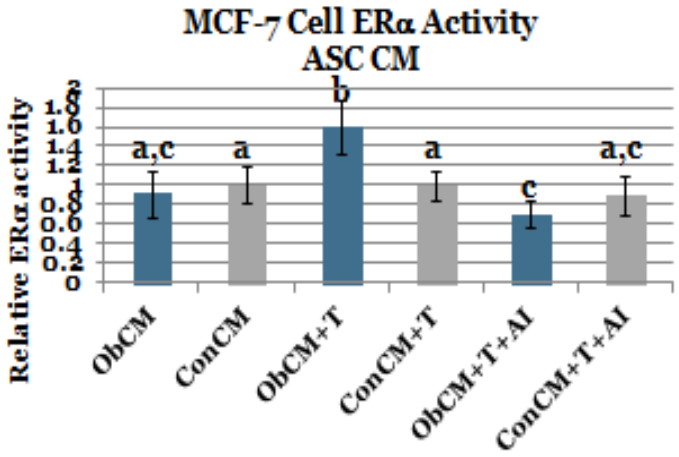
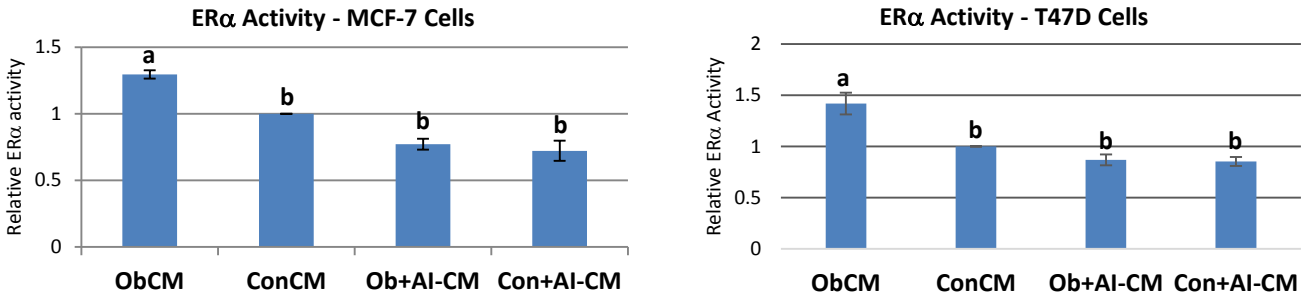


Figure 18. MCF-7 and T47D cell ERα activity, measured by ERE luciferase assay, following a 48 hour exposure to CM from a co-culture of U937 cells (first differentiated to macrophages) and ASC. This CM was generated by exposing the co-culture to Ob or Con human sera for 1 hour, removing the serum and washing the cells with PBS, then incubating the co-culture in SFM plus exogenous testosterone (ObCM and ConCM) or testosterone and the aromatase inhibitor anastrozole (Ob+AI-CM and Con+AI-CM) for 24 hours. The SFM (now CM) was then collected and used to treat the MCF-7 or T47D cells. Different letters indicate significant differences ($p<0.05$).



Appendix B: Poster Abstracts

NSAID use attenuates breast cancer recurrence in obese women: Role of prostaglandin-aromatase interactions. *Bowers LW, Maximo IXF, Tekmal RR, Hursting SD, Beeram M, Brenner AJ, deGraffenried LA.* 36th Annual CTRC-AACR San Antonio Breast Cancer Symposium, 2013.

Introduction: Obesity is associated with a worse breast cancer prognosis, with the most prominent effects seen in hormone responsive postmenopausal patients. It has also been linked to elevated levels of inflammation, including greater cyclooxygenase-2 (COX-2) expression and activity in adipose-infiltrating macrophages. The product of this enzyme, the pro-inflammatory eicosanoid prostaglandin E2 (PGE2), stimulates adipose tissue aromatase expression and subsequent estrogen production, which could promote breast cancer progression. Consequently, we hypothesized that non-steroidal anti-inflammatory drug (NSAID) use decreases estrogen receptor (ER) positive breast cancer recurrence in the obese population via inhibition of PGE2-mediated local aromatase expression.

Methods: Four-hundred and forty women treated for invasive, ER positive breast cancer at San Antonio area clinics were retrospectively classified according to NSAID use, body mass index category (Normal Weight

(NW): 18.5-24.9 kg/m2; Overweight (OW): 25.0-29.9 kg/m2; Obese (OB): ≥30.0 kg/m2), and disease recurrence. To examine the role of obesity-induced local aromatase expression in the link between NSAID use and disease recurrence, we utilized an *in vitro* model of obesity in which we exposed macrophages to pooled sera samples from NW or OB postmenopausal breast cancer patients. Adipose stromal cells' (ASC) aromatase expression was measured following exposure to conditioned media (CM) collected from these sera-exposed macrophages. ER activity and *in vitro* measures of cancer aggression were assessed in MCF-7 and T47D breast cancer cells cultured in CM from sera-exposed macrophage/ASC co-cultures.

Results: Within our patient population, which had an average BMI in the obese range, NSAID users had significantly lower recurrence rates ($p=0.05$) and their time to disease progression was delayed by almost 28 months in comparison to nonusers. Our *in vitro* studies demonstrated that growth in OB macrophage CM significantly enhances ASC aromatase expression in comparison to NW ($p<0.05$), while macrophage treatment with celecoxib during the generation of CM neutralizes the difference between OB and NW. This was correlated with significantly greater macrophage PGE2 production following OB versus NW sera exposure ($p<0.05$). In addition, CM from macrophage/ASC co-cultures exposed to OB patient sera stimulates more breast cancer cell ER activity, proliferation, and S phase activity, and these differences are eliminated by the addition of an aromatase inhibitor during the generation of CM. We also plan to examine how the co-culture CM impacts breast cancer cell expression of a panel of genes related to cancer aggression.

Conclusions: Our results indicate that NSAID use can improve the recurrence rate for hormone-responsive breast cancer patients, particularly those with an elevated BMI. The *in vitro* model suggests that obesity-related enhancement of PGE2-induced local aromatase expression and estrogen production may be a key mechanism mediating this effect. Further studies designed to examine the clinical benefit of NSAID use in the obese breast cancer patient population are warranted.

Obesity promotes pre-adipocyte aromatase expression via breast cancer cell prostaglandin E2 production in an in vitro model of the breast tumor microenvironment. *Bowers LW, Brenner AJ, Hursting SD, deGraffenried LA.* American Institute for Cancer Research Annual Research Conference, Bethesda, MD, 2013.

Background: Obesity is associated with a worse breast cancer prognosis, particularly in estrogen receptor (ER) positive, postmenopausal patients. Resistance to aromatase inhibitor treatment may be one mechanism mediating this effect, as obese patients have a lower response rate to this class of drugs and higher breast tissue aromatase expression. We hypothesized that obesity-associated circulating factors promote breast cancer cell COX-2 expression and prostaglandin E2 (PGE2) production, resulting in an elevation in preadipocyte aromatase expression that enhances breast cancer cell estrogen receptor activity and proliferation.

Methods: We utilized an *in vitro* model of the obese patient's tumor microenvironment in which cultured MCF-7 breast cancer cells and preadipocytes were exposed to pooled serum from obese (OB; BMI≥30.0 kg/m^2) or normal weight (N; BMI 18.5-24.9 kg/m^2) postmenopausal women.

Results: Exposure to OB patient sera increased MCF-7 cell PGE2 production by 14-fold versus N, independent of any change in COX-2 expression. This was coupled with 65% greater preadipocyte aromatase expression following culture in conditioned media (CM) from MCF-7 cells exposed to OB versus N patient sera, a difference nullified by treatment of the MCF-7 cells with the COX-2 inhibitor celecoxib during CM generation. Analysis of the sera revealed significantly higher interleukin 6 (IL-6) concentrations in the OB versus N patient samples. Depletion of IL-6 from the sera resulted in neutralization of the difference between OB and N CM-stimulated aromatase expression. In addition, CM from pre-adipocyte/MCF-7 cell co-cultures exposed to OB patient sera stimulated greater MCF-7 cell ER activity and proliferation compared to N.

Conclusions: This study indicates that obesity-associated, breast cancer cell-derived PGE2 induces preadipocyte aromatase expression, thereby promoting greater cancer cell ER activity and proliferation. Investigation regarding the efficacy of a COX-2 inhibitor/aromatase inhibitor combination therapy in the obese postmenopausal patient population is warranted.

Obesity promotes elevated aromatase expression in the breast tumor microenvironment via upregulation of prostaglandin E_2 production. *Bowers LW, Li R, Tekmal RR, Brenner AJ, Hursting SD, deGraffenried LA.* Keystone Symposia: The Role of Inflammation during Carcinogenesis, Dublin, Ireland, 2012.

According to clinical correlation studies, obesity is associated with a worse breast cancer prognosis for both pre- and postmenopausal women. One mechanism for this negative effect may be endocrine therapy resistance, as obese postmenopausal patients' response rate to aromatase inhibitors is significantly lower than lean patients'. Obesity is accompanied by elevated levels of circulating cytokines and growth factors that can promote epithelial cell prostaglandin E_2 (PGE_2) production via COX-2, and PGE_2 is a known regulator of adipose stromal cell (ASC) aromatase expression. Consequently, we hypothesized that exposure to obesity-associated circulating factors increases breast cancer cell production of PGE_2, resulting in greater local ASC aromatase expression.

To test this hypothesis, we utilized an *in vitro* model of obesity in which we exposed MCF-7 breast cancer cells to pooled serum samples from obese (BMI≥30.0 kg/m^2) or lean (BMI 18.5-24.9 kg/m^2) postmenopausal women. Following serum exposure, the cells were incubated in serum-free media for 24 hours. We then examined the effect of the resulting MCF-7 conditioned media (CM) on ASC aromatase expression, finding that obese CM produced >50% higher expression in the ASC than lean CM. Preliminary data also indicates that PGE_2 levels in the obese CM are approximately ten-fold greater than lean CM. Measurement of tumor necrosis factor alpha (TNF-a), interleukin 6 (IL-6), and insulin-like growth factor 1 (IGF-1) concentrations in the serum samples revealed that all were higher on average in the obese versus lean subjects.

To expand on these findings, we plan to assess the role of these circulating factors as well as COX-2 signaling in the production of the breast cancer cell-mediated upregulation of ASC aromatase expression by depleting the obese serum of each factor and utilizing a COX-2 inhibitor. Estrogen production by the ASC following CM exposure will also be measured to assess its correlation with ASC aromatase expression. Through greater understanding of the signaling pathways responsible for obesity's upregulation of aromatase, we hope to identify molecular targets that will lead to a more effective chemotherapeutic regimen for the obese postmenopausal population.

Obesity-associated growth factor signaling promotes aromatase expression and estrogen receptor activity in breast cancer cells. *Bowers LW, De Angel R, Brenner AJ, Tekmal RR, Hursting SD, deGraffenried LA.* 102nd Annual Meeting of the American Association for Cancer Research, Chicago, IL, 2012.

Obesity increases the risk of breast cancer by approximately 50% in postmenopausal women and is also associated with a worse prognosis. Elevated estrogen synthesis by the local mammary epithelia and adipose tissue is thought to be the principal mediator of breast tumorigenesis in this population, which primarily develops estrogen receptor alpha (ERa) positive breast cancer. However, the elevated levels of free insulin-like growth factor 1 (IGF-1) that accompany obesity are also thought to play a role. IGF-1 has significant tumorigenic effects in the breast and also regulates aromatase, the key enzyme in the conversion of androgens to estrogen. Consequently, we hypothesized that obesity increases the risk of postmenopausal breast cancer via growth factor-induced aromatase expression and/or activity in the local mammary tissue.

We have previously shown that MMTV-Wnt-1 mammary tumors from obese ovariectomized mice express higher levels of aromatase in comparison to tumors from lean ovariectomized mice. To examine the molecular pathways responsible for this effect, we have utilized an *in vitro* model of obesity in which ERa positive MCF-7 breast cancer cells were exposed to human sera obtained from postmenopausal women and pooled by BMI category . Our data indicates that exposure to obese (BMI ≥ 30 kg/m^2) human serum stimulates greater aromatase expression in comparison to control (BMI: 18.5-24.9 kg/m^2). This is coupled with enhanced ERa activity when exogenous androgen is present, indicating that the increased aromatase expression results in greater estrogen production. Obese human sera also activates MCF-7 cells' Akt pathway to a greater degree

than control, while MCF-7 cells expressing a constitutively active Akt demonstrate higher levels of aromatase expression in comparison to MCF-7 cells. This suggests that circulating growth factors in the obese sera, like IGF-1, may induce elevated aromatase expression via the downstream PI3K/Akt pathway.
To expand on our findings, we plan to assess whether treatment with PI3K/Akt pathway inhibitors eliminates the difference in MCF-7 cells' aromatase expression following exposure to obese versus control sera. We will also examine how inhibition of the PI3K/Akt pathway affects ERa activity in sera-exposed MCF-7 cells. Through elucidation of the signaling pathways responsible for obesity's upregulation of local aromatase expression, we ultimately hope to develop rational and effective chemopreventive and chemotherapeutic regimens for the high-risk obese postmenopausal population.

Obesity-induced aromatase expression in the breast microenvironment promotes estrogen receptor activity independent of circulating estradiol levels. *Bowers LW, Brenner AJ, Li R, Tekmal RR, deGraffenried LA.* 34th Annual CTRC-AACR San Antonio Breast Cancer Symposium, 2011.
Epidemiological studies indicate that obesity increases the risk of postmenopausal breast cancer by approximately 50%. In the past, researchers have hypothesized that elevated estrogen synthesis by the peripheral adipose tissue may be the principal mediator of breast tumorigenesis in this population, which primarily develops estrogen receptor alpha (ERα) positive breast cancer. However, obesity is also accompanied by an elevation in growth factor and cytokine signaling, and these pathways have been linked to tumorigenesis. In addition, certain growth factor and cytokine family members can promote aromatase expression in both the epithelial and stromal tumor compartments. Consequently, we hypothesized that obesity increases the risk of postmenopausal breast cancer via elevated aromatase expression and/or activity in the local mammary tissue.
To test our hypothesis, we investigated how ERα activity in mammary epithelial cells was influenced by adipose stromal cells (ASC) cultured under obesity-associated conditions, including high cell density and exposure to elevated levels of circulating growth factors and cytokines. For the latter condition, sera was obtained from postmenopausal women, pooled by BMI category (lean: 18.5-24.9; obese: ≥30), and applied to the ASC, which were originally derived from women undergoing reduction mammoplasty. High ASC density was achieved via the protocol previously published by Dr. Li. Preliminary data indicated that both elevated cell density and sera from obese postmenopausal women induces greater aromatase expression in ASC, indicating that multiple factors may be contributing to the increased local aromatase expression seen with obesity. We are currently exploring the signaling pathways responsible for obesity's upregulation of ASC aromatase expression and will present these results at the meeting. Intriguingly, exposure to conditioned media from both the high density and obese sera-exposed ASC enhanced ERα activity in MCF-7 mammary epithelial cells, independent from exogenous estradiol but dependent on the presence of androgens, suggesting an important role for the aromatase enzyme in this observation. To expand on this finding, we plan to investigate the effect of ASC conditioned media on different markers of cancer aggression, including proliferation and survival, and assess the degree to which these effects depend on estradiol. Through further examination of obesity's impact on signaling pathways in both the epithelial and stromal tumor compartments, we ultimately hope to identify more effective chemopreventive and therapeutic regimens for the high-risk obese postmenopausal population.

Obesity-associated growth factor signaling upregulates aromatase expression in breast cancer cells. *Bowers LW, De Angel R, Brenner AJ, Hursting SD, deGraffenried LA.* 101st Annual Meeting of the American Association for Cancer Research, Orlando, FL, 2011.
According to epidemiological studies, obesity increases the risk of breast cancer by approximately 50% in postmenopausal women. Elevated estrogen synthesis by the adipose tissue and local mammary epithelia is thought to be the principal mediator of breast tumorigenesis in this population, which primarily develops

estrogen receptor alpha (ERα) positive breast cancer. However, obesity-associated growth factor and cytokine signaling are also thought to play a role. Specifically, obesity is accompanied by elevated levels of insulin, insulin-like growth factor 1 (IGF-1), and leptin. These all have significant tumorigenic effects in the breast and are known to regulate aromatase, the enzyme that catalyzes the conversion of testosterone to estradiol. Consequently, we hypothesized that obesity increases the risk of postmenopausal breast cancer via growth factor-induced aromatase expression and/or activity in the local mammary tissue.

MMTV-Wnt-1 transgenic (Wnt-1 TG) mice, which are predisposed to the development of estrogen receptor positive mammary adenocarcinoma, were ovariectomized to mimic a postmenopausal state. They were then randomized to either a diet-induced obesity (DIO) chow (60% calories from fat) or control chow (10% calories from fat) to produce an obese or lean phenotype, respectively. qPCR analysis revealed that aromatase expression in the mammary tumors of the obese mice was almost four-fold greater than in the controls. To examine the molecular pathways responsible for this effect, we have utilized an *in vitro* model of obesity in which ERα positive MCF-7 breast cancer cells were treated with sera from C57BL/6 mice fed either the DIO or control diet. Human sera obtained from women involved in a case-controlled clinical trial will be pooled by BMI category and also used to treat the cells. Preliminary data indicates that obese mouse sera activates the PI3K/Akt pathway to a greater degree than lean mouse sera. Our lab has also shown that MCF-7 cells expressing a constitutively active Akt demonstrated 37% greater aromatase expression in comparison to vector-transfected MCF-7 cells. This suggests that circulating growth factors in the obese sera may induce elevated aromatase expression via the PI3K/Akt pathway.

These results will be further explored by measuring aromatase expression and aromatase promoter activity in response to treatment with sera from obese and lean subjects. To determine the role of obesity-associated growth factor and leptin signaling on aromatase expression and activity, we will also add inhibitors to these different pathways with the sera treatment. Through elucidation of the signaling pathways responsible for obesity's upregulation of aromatase, we hope to lay the foundation for future research that could result in an effective chemopreventive regimen for the high-risk obese postmenopausal population.